



IMPROVEMENT OF THE ATOMIC ABSORPTION SPECTROMETRY METHOD

Determination of calcium and magnesium
from human urine

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ABSTRACT

Tampereen ammattikorkeakoulu
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Improvement of the Atomic Absorption Spectrometry Method –
Determination of Calcium and Magnesium from Human Urine

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This Bachelor's thesis was conducted at Tampere University of Technology at the Department of Chemistry and Bioengineering in summer 2011. The main aim of the research group was to study the means of preventing odours of the urine and to study and possibly prevent the precipitations occurring in urine while it is stored.

This thesis focused on the study of precipitations and especially on the improvement of the methods of measuring the amounts of calcium and magnesium in the urine's liquid phase at two different time periods. The chosen equipment to study the amounts of these metals was atomic absorption spectrophotometer. The suitability of the method was tested by using the chosen validation factors. The main factors were the yield, linearity and repeatability of the method.

The initial method which was in use proved to be inefficient and the obtained results stayed under the imposed limits. An intensive literature survey indicated that the methods needed some modifications. All improvements for the methods were not found from the literature however, so some new means were needed.

The improvements of the methods were very satisfying and the results reached the imposed limits. Particularly good improvements were obtained with the yield, as the recovery arose from the level of 80% into the level of 100%. At the same time, all the other measured factors either stayed in the previous, good levels, or the results were improved.

Overall, the improvements of the methods were really successful and the created methods were taken into use. The results obtained with improved methods were now proved to be on a reliable level, and therefore the results could be presented forward. If the method is to be used more widely in the future, the use of more comprehensive validation is recommended.

Key words: Method improvement, atomic absorption spectrophotometry, interferences, yield, precipitations.

TIIVISTELMÄ

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Tämä opinnäytetyö tehtiin Tampereen teknillisellä yliopistolla, kemian ja biotekniikan laitoksella kesällä 2011. Työ tehtiin EU-rahoitteisessa DryCloset-nimisessä tutkimusryhmässä, jonka päätavoite oli poistaa hajuja virtsasta, ja tutkia sekä mahdollisesti estää virtsaan säilytyksen aikana muodostuvia saostumia.

Tämä opinnäytetyö keskittyi lähinnä syntyvien saostumien tutkimiseen. Saostumien muodostumista tutkittiin määrittämällä nestefaasin magnesium- ja kalsiumpitoisuuksia virtsasta säilytysajan alku- ja loppuvaiheessa. Metallien pitoisuudet analysoitiin kyseisinä ajankohtina atomiabsorptiospektrofotometrillä. Laite ei ollut täysin tuttu kaikille ryhmän jäsenille ja siten käyttäjille, joten sen käytön opastus tuli yhdeksi osaksi opinnäytetyön tavoitetta. Lisäksi jo käyttöön otettujen mittausmenetelmien sopivuutta haluttiin testata valittujen validointitekijöiden avulla, erityisesti saannon haluttiin yltävän hyvälle tasolle.

Laaja kirjallisuuskatsaus ja ensimmäiset suoritettavat mittauskokeet osoittivat, että menetelmien kehittäminen on tarpeen. Menetelmille asetettiin tietyt raja-arvot joihin pyrittiin, jotta voitiin sanoa tulosten yltävän halutulle tasolle. Raja-arvojen saavuttamista testattiin valittujen validointitekijöiden avulla. Suuri haaste menetelmän kehittämisessä oli erityisesti virtsan monimutkainen matriisi ja sen aiheuttamat häiriötekijät.

Menetelmien kehitystyö oli lopulta onnistunut ja monilta osin saavutettiin erittäin hyvin ennalta-asetetut tavoitearvot. Erityisen tyytyväisiä oltiin saannon parantumiseen menetelmien kehittämisen ansiosta. Saantotestissä tulokset saatiin nousemaan 80%:sta hyvin lähelle tavoiteltua 100%:a ja samalla muut mitatut tekijät pysyivät samoissa hyvissä lukemissa tai paranivat hieman alkuperäisellä menetelmällä saavutetuista tuloksista.

Työtä ja sen tuloksia voidaan pitää hyvin luotettavina ja siksi tuloksia voidaan välittää eteenpäin yhteistyökumppaneille. Tulevaisuudessa laajemmän ja kunnon validoinnin suorittamista useammilla tekijöillä ja toistoilla voidaan suosittaa tehtäväksi, etenkin jos menetelmä otetaan laajempaan käyttöön.

Avainsanat: Menetelmän kehitys, atomiabsorptiospektrofotometri, häiriötekijät, saanto, saostumat

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1 INTRODUCTION

Struvite (magnesium ammonium phosphate hexahydrate, ($NH_4MgPO_4 \times 6H_2O$)) is a phosphate mineral, which precipitates in alkaline urine. During the storage of urine, the urea in it breaks down into ammonium and the pH of the solution starts to rise. This causes problems in dry toilet systems because the precipitates form clogs in the tanks and pipelines. In waterless toilets, this problem only increases with the time. That is why the struvite and other precipitates are a matter of great importance for closer investigation.

The precipitations of struvite and octacalcium phosphate (OCP) are mainly caused by the amount of metals and the rising pH of urine. According to the previous studies, the levels of magnesium and calcium were found to be the most limiting factors for the amount of precipitates. Therefore, the levels of magnesium and calcium in fresh and stored urine were chosen as study subjects. The best equipment available for these tests was the flame atomic absorption spectrometer (AAS). The concentrations of magnesium and calcium were studied from the liquid phase of the sample. The result of these analyses can indicate how much phosphate salts have precipitated within the studied time period.

Human urine was collected from voluntary donators and stored in a controlled environment. The levels of pH, conductivity and ammonia were observed regularly from the samples. The AAS measurements were performed in the beginning and at the end of the storage period.

This Bachelor's thesis was conducted at Tampere University of Technology (TUT) in the Department of Chemistry and Bioengineering. The research has received funding from the European Union's Seventh Framework Programme managed by REA-Research Executive Agency <http://ec.europa.eu/research/rea> ([FP7/2007-2013][FP7/2007-2011]) under grant agreement n° [256295]. The project was called Drycloset and its main aim was to remove the odours of urine and to study and possibly prevent the precipitation of struvite and other precipitates in urine. The aim of the study was to create the optimal method for magnesium and calcium measurements from the urine and to create a user's manual for this method.

Similar studies had not been previously conducted in TUT, so the method had to be tested and improved. This was done by using certain validation factors with limits checked from previous struvite studies or decided by the group.

2 DRY TOILETS

One of the human basic needs in everyday life is a working toilet. In every case, it is not necessary to spend tons of water to have well functioning hygienic toilets. The technology used in dry toilets offers a variety of different choices to use. The use of dry toilets saves pure drinking water, helps the waste water management, collects nutrients, and does not pollute ground water. Nowadays, there are many suitable dry toilet models to be found in different places with good maintenance and user comfort. Good ventilation is an important factor for the function of dry toilets and it can remove all developed odours from toilets. (Global Dry Toilet Association of Finland 2011.)

A person will produce on average about 500 litres of urine and 50 kg faeces yearly. These (mostly urine) include some nutrients, which can also be utilised as fertilisers. (Global Dry Toilet Association of Finland 2011.) Dry sanitation is in other words the disposal of human waste without the use of water as a carrier. The waterless toilets are water and chemical free, so they save water and protect the environment among other things by decreasing the amount of wastewater. In dry toileting, the final waste can be destroyed by burning, burying, composting or by land filling. Dry sanitation has clear environmental, social, financial and public health advantages. It is a good option to be used especially in developing countries. (Solar San 2005.)

3 COMPOSITION OF URINE

Normally, humans excrete about 600–2,500 ml urine per 24 hours. The exact daily amount varies depending on water intake, external temperature and perspiration, the individual's mental and physical state, and also on diet. Most of the daily volume of urine is formed during sleep and only half as much urine is formed during the activity. That is the reason why morning urine is normally the most concentrated. Urine has its specific gravity which normally ranges from 1.003-1.030, depending on the variations of solute concentrations in the urine. (Harper, Rodwell & Mayes 1979.)

Usually around 95% of the urine is water and all the other components are dissolved in it. Most of these other substances are metabolic wastes which include nitrogen. Urine contains some inorganic and organic compounds of sulphur and a very small amount of proteins are also found in it. Overall, urine contains thousands of different substances, but the amount of each of these is very small. (Nienstedt, Hänninen, Arstila, Björkqvist 1991.)

The chief pigment of the urine is urochrome, which normally gives the pale yellow or amber colour to the fresh urine. Some drugs or illnesses can transform the colour. (Harper etc. 1979.) During storage, the colour of urine can get muddy, because of increased amount of bacteria and precipitation of some compounds. (Nienstedt etc. 1991).

Fresh urine is usually acidic with the pH under 6. This is caused by the phosphates and sulphates which are produced in the catabolism of proteins. During storage, urine becomes alkaline because the urea is converting to ammonia, and at the same time CO₂ is being released into air. (Harper etc. 1979.) Urine's grade of acidity, or pH value, depends on the acidic and alkaline products of metabolism. Meat products increase acidity, and fruits in turn increase alkalinity. Normal pH can vary between the values of 4.6 and 8.2. (Nienstedt etc. 1991.)

Urea covers about half of urine's normal constituents, and sodium chloride and its derivatives cover about one fourth. The excretion of urea is directly related to the intake of proteins and it is the principal end product of the mammal protein metabolism. From the total urinary nitrogen around 80–90% is in the form of urea. (Harper etc. 1979.) Nitrogen breaks down into ammonia and then via nitrification to nitrite and finally to

nitrate (Heinonen-Tanski, Pradhan & Karinen, 2010). Some diseases, for example diabetes as well as high protein catabolism, can raise the amount of urea in urine. The amount of ammonia in fresh urine is usually low, but after standing it can increase because of the breakdown of nitrogen compound. Some diseases can also increase or decrease the amount of ammonia in fresh urine. (Harper etc. 1979.)

Uric acid is the most important end product of the oxidation of purines (nucleotides) in the human body. The amount of uric acid depends on the diet and the breakdown of cellular nucleoproteins in the body. Uric acid is only slightly soluble in water, but it forms easily some soluble salts with alkali. Because of the salt formation, uric acid precipitates on standing urine, while pH decreases. (Harper etc. 1979.)

The amount of daily excreted amino acid nitrogen is around 150-200 mg for adults, and for children the amount is even higher. Even though the renal threshold for these substances is quite high, all naturally occurring amino acids have been found in human urine. High percentages of excreted amino acids are in a combined form and will be released by acid hydrolysis. Some are also in the form of free amino acids. (Harper etc. 1979.)

Phosphates in urine are combinations of sodium and potassium phosphates as well as calcium and magnesium phosphates. Calcium and magnesium may form precipitates in alkaline urine. Diet influences the concentration of excreted phosphate, particularly the content of proteins, the most. The amount of oxalates is usually very low in urine. Sodium chloride is the main chloride type in urine, and its output varies considerably with intake. (Harper etc. 1979.)

In normal urine, the quantities of hormones, vitamins and enzymes are quite small. The urinary content of these substances is still often in an important role in diagnostic measurements. Sodium, potassium, calcium, and magnesium are the main minerals present in urine. (Harper etc. 1979.) The amounts of magnesium and calcium in urine were measured at TUT with AAS equipment from fresh and stored urine. The results from the measurements are introduced in table 1.

TABLE 1 Measured magnesium and calcium concentrations in fresh and stored urine in different pH, measurements conducted at TUT by the group Drycloset.

	Fresh combined urine	Stored combined urine
pH	~5.8	~9.0
Magnesium	60-125 mg/l	~0.2 mg/l
Calcium	55-125 mg/l	~2 mg/l

4 STRUVITE

4.1 Struvite and its precipitation

Magnesium ammonium phosphate hexahydrate ($NH_4MgPO_4 \times 6H_2O$), also known as struvite, is a white inorganic crystalline mineral, which can cause blockages in dry toilets and other wastewater systems by precipitating in pipelines and collecting tanks. The precipitations of struvite have been successfully removed by acid washing or manually with a hammer and chisel. However, these techniques are not the most ideal options to be used, because they are time consuming and complex to carry out. (Stratful, Scrimshaw & Lester 2001.) Several other processes than ureolysis and precipitation also influence the occurrence and extent of inorganic blockages in urine-collecting systems. The other factors are the dilution of urine, the retention time, and foreign solids. (Udert, Larsen & Gujer 2003b.) Struvite formation causes maintenance problems in dry toilets, especially when they are in active use for many years.

Struvite consists of equal molar concentrations of magnesium, ammonium and phosphorus. The general reaction of struvite forming is shown in formula 1. However, the chemistry of struvite precipitation is not as simple as the equation shows (Doyle & Parsons 2002.) The precipitation is studied mostly with wastewater, but the main theory is practically the same with urine.



Struvite is the main precipitating subject from urine, but also other substances have been found to precipitate, like octacalcium phosphate OCP, hydroxyapatite (HAP), and calcite ($CaCO_3$). HAP and calcite precipitations are found to precipitate only when the urine has been diluted with tap water, so they do not occur in dry toilets. (Udert, Larsen, Biebow & Gujer 2003a).

The precipitates, both struvite and OCP, are limited by the amount of calcium and magnesium. The amount of magnesium and calcium specify the composition of solids, which will affect the composition of precipitates. The precipitation of calcium and

magnesium phosphates is quite a fast process. The precipitation of struvite will be completed faster, while the OCP precipitates more slowly. All these precipitations cause a risk of blockages especially in the pipes of toilets where they accumulate. Precipitates in pipes are frequent and persistent. (Udert etc. 2003a.)

4.2 Features of struvite

The formation of struvite includes two stages. In the first stage, the constituent ions form crystal embryos, which will grow in the second stage, until the equilibrium is reached. In the places where struvite constituents are continuously replenished, the crystal growth can be indefinite, like in urine collecting tanks and pipelines. Usually the crystal formation occurs spontaneously, but it can also be aided by the presence of suitable nuclei, which can either be solid impurities in suspension or on the sites of the pipelines. (Doyle etc. 2002.)

The solubility of struvite is mostly defined by the concentrations of magnesium, ammonium and phosphate, but the pH of the solution has an influence on it too. (Doyle etc. 2002). The temperature also has an impact on struvite solubility, and the maximum solubility for struvite has been proved to be at +50 °C. After that, the solubility begins to fall. The temperature tests have been conducted at temperatures of 10 to 65 °C. (Aage 1997, according to Doyle etc. 2002). With the increasing pH, the struvite solubility is indicated to be decreasing. While the pH is under 8, the precipitation of struvite is fairly slow. (Doyle etc. 2002.)

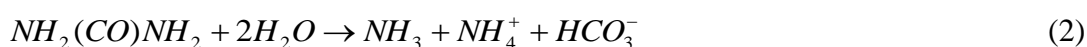
Uderts etc. (2003b) studies prove that precipitation is faster due to a faster pH increase. In the introduced results, the dissolved magnesium was precipitated 99% during the experiment, the calcium precipitation was 70%, and precipitation for phosphate was 44%. The precipitation of struvite is already possible at a low supersaturation rate, while the other precipitations like hydroxyapatite and calcite need higher supersaturation to occur. (Udert etc. 2003b.)

The precipitation of struvite may be controlled by pH, degree of supersaturation, temperature, and the presence of other ions in the solution. Also the inhibition of formation is one option to control struvite precipitation (Doyle etc. 2002.) Inhibition

may be performed with chemicals, like Buchanan (1994) has indicated with ferric chloride (Doyle etc. 2002). Emsley (2000) has reported sodium hydrogen diphosphate and sodiumpolyphosphate to inhibit struvite precipitation by making magnesium unavailable to precipitate. (Doyle etc. 2002).

5 UREASE

Ureases (urea aminohydrolases) are highly proficient enzymes which catalyse the hydrolysis of urea, giving rise to the final product of carbonic acid and ammonia. The overall reaction is shown in formula 2. Ureases are found widely from nature, for example from plants, bacteria and fungi. Although they have different protein structures, the function is the same. A common feature of the enzymes is the presence of metals in their active sites. The task of these metals is to activate the substrate and water for the reaction. (Krajewska 2009).



Normally, ureases will act best in a neutral environment, and they are known to be very pH-dependent enzymes. The optimum pH for urease to be active is at pH 7–8. However, they can operate on as wide a scale as pH 4.5–10.5. (Krajewska 2009.) The release of the ammonia will increase pH during ureolysis. (Udert etc. 2003b). In human body, the activity of ureases can lead to the formation of urinary stones, which consist of struvite and carbonate apatite – the same products which precipitate from the action of ureases in the process of ureolysis. (Krajewska 2009). In environmental conditions, the urease synthesis may be regulated by the availability of nitrogen sources, urea concentration, or the pH. (Udert etc. 2003b).

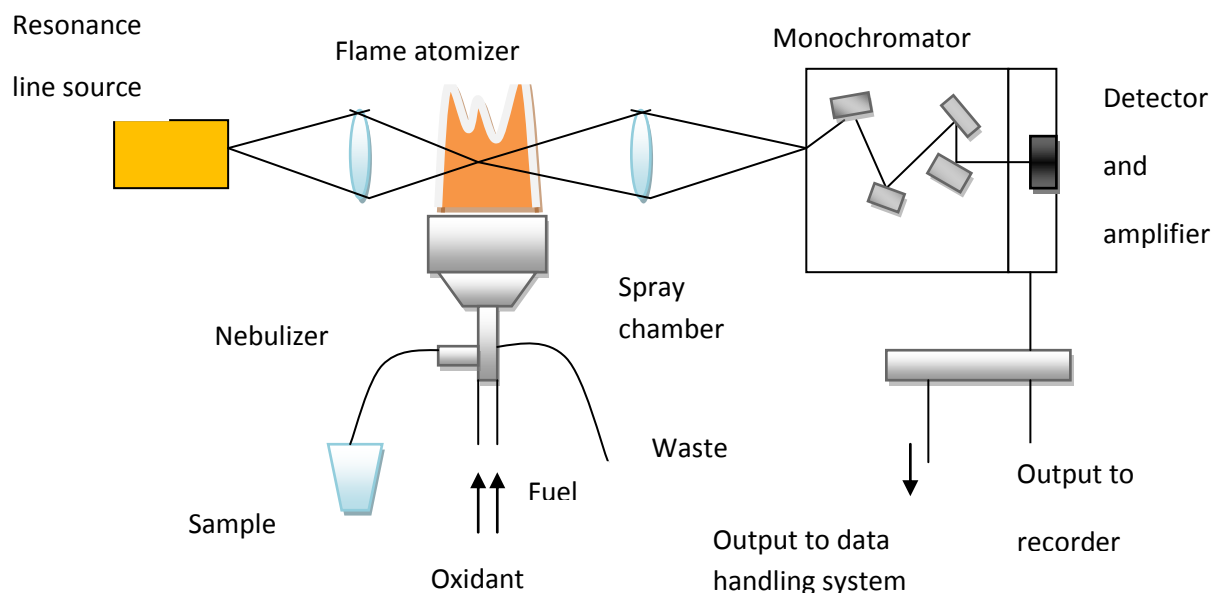
Ureolysis is usually a microbial process, which triggers the precipitation of struvite. The microbial ureolysis is a very fast process, and therefore only a small amount of urea has to be hydrolysed for substantial precipitation to occur in a short retention time. The bacterial ureolysis usually follows the Michaelis-Menten kinetics, although the kinetic constant can vary widely from species to species. Michaelis-Menten kinetics reflects the dependence of reaction time to the content of the substrate. (Udert etc. 2003a.)

6 THEORY OF ATOMIC ABSORPTION SPECTROSCOPY

The main operating idea of the atomic absorption spectroscopy (AAS) is to decompose the sample into atoms by vaporising it in high temperature, and then to measure the characteristic wavelength of radiation to obtain the correct atom concentrations. The atomic absorption spectroscopy is a very sensitive method and it can be used to analyse a specific analyte in a complex sample. The analyte is measured at parts per million to parts per trillion levels, and samples with high concentrations must be diluted to reach these levels. (Harris 2007.)

6.1 Basic components and their function in AAS

The basic requirements for flame atomic absorption spectroscopy analysis are the fuel-oxidant flow, the flame, the lamp which is the hollow cathode lamp or electrodeless discharge lamp (EDL), the sample in a liquid form, the monochromator, the amplifier, and the detector, as well as the readout device, which is usually a computer with software. (Harris 2007.) The main components of AAS equipment are visualised in picture 1.



PICTURE 1. The main components of an atomic absorption spectrometer. Modified from picture Lajunen & Perämäki (2004) by Mäkinen H.

The lamp works as a radiation source by emitting a sharp line spectrum characteristic of the analyte element. Then the emission beam coming from the radiation source is modulated, and this modulated signal passes through the atomic vapour where the atoms of the analyte absorb radiation. The monochromator selects the desired spectral line, after which the isolated analyte lines fall onto the detector, which gives the electric signal converted from the light signal. The signal is amplified by a selective amplifier and the signal is recorded with the computer or other readout device. (Lajunen & Perämäki 2004.)

6.2 Atomisation of the sample

In atomic absorption spectrometry, the analyte can be atomised by a flame, an electrically heated furnace, or plasma. The flame technique is the oldest, but the inductively coupled plasma and the graphite furnace have replaced it in many laboratories. Nevertheless, the flame technique is still mostly used in teaching laboratories. (Harris 2007.)

The most common fuel-oxidiser combination is acetylene and air. With this setting, the temperature of the flame is around 2400–2700 K. When a hotter flame is needed, the acetylene is used with nitrous oxide, which gives a temperature of 2900–3100 K. The chosen fuel-oxidiser combination and the sample are mixed together in a premix burner and then introduced into the flame. The sample liquid breaks into a fine mist and finally turns into an aerosol, a fine suspension of liquid particles in a gas, inside the premix burner. Only 5% of the initial sample is left in the aerosol, which eventually reaches the flame. When the droplets enter the flame, they evaporate and the remaining solid vaporises and decomposes into atoms. Depending on the measured element, the flow of the sample, fuel and the oxidiser, the maximum atomic absorption can be reached and the different height of the flame observed. (Harris 2007.)

The furnace is a more sensitive method and it requires less sample material than the flame. The use of the furnace requires more operator skills to find the proper conditions for each type of sample. The furnace is heated in steps and it needs to be purged with argon or nitrogen gas between every sample. By using the furnace method, it is possible to analyse soluble samples directly. (Harris 2007.)

Inductively coupled plasma is twice as hot as the flame with a temperature around 6000–10000 K. Argon is used as a coolant gas. There are fewer interferences than with the flame method because of the high temperature, stability, and relatively inert argon environment. However, the costs of purchasing and operating the plasma are higher than with the flame. (Harris 2007.)

In atomic absorption spectrometry, the temperature influences the strength of the observed signal, because it determines the degree of sample breakdown into atoms. The extent of the atoms' ground, excited, and ionised states varies in different temperatures. The temperature is more critical when using the atomic absorption method than with the atomic emission technique. (Harris 2007.)

6.3 Light and lamp in AAS

For every analysis, the correct lamp is chosen according to the studied subject. The lamp transmits light in a short wavelength area. In this area, the examined subject will absorb the light. The emission of the lamp is chosen according to the examined subject and the other subjects in the sample will not cause any absorption in these conditions. This makes the AAS method a very selective one. (Jaarinen & Niiranen 2005.)

The light of the lamp is led to the flame or graphite oven, where the intensity decreases according to Lambert-Beer's law, which means that the absorption of the electromagnetic radiation depends on the amount of absorbing subject exponentially. The monochromator is located after the opening for samples, and its purpose is to remove other wavelengths than the one used in analysis. In order to differentiate the measuring light from other lights than the sample emits, it is modulated. The absorbance of the equipment itself is brought to zero by a liquid which has the same background as the samples. (Jaarinen & Niiranen 2005.)

6.4 Interferences in AAS method

Every effect that changes the signal in the analysis, while the analyte remains unchanged, is called interference. Many of them can be corrected by removing the source of the interference or making the standards identical with samples, so that they contain the same interference factors as the samples. (Harris 2007.) In other words, the interferences are caused by differently behaving samples and reference solutions while performing the measurements. The basic idea of the AAS measurement is the comparison of the signal produced by the sample solution against the signal caused by the reference solution. (Lajunen & Perämäki 2004.)

6.4.1 Chemical interferences

Some components in the sample can decrease the extent of atomisation of the analyte (Harris 2007). These are known as chemical interferences, which originate either in the flame or in the sample solution. In these situations, the atomisation of the analyte may not be complete or other atoms and radicals, which are present in the gas phase, react with vaporising atoms. The analyte element may also form new compounds, which possess different thermochemical characteristics and will therefore behave in a different way. (Lajunen & Perämäki 2004.)

The chemical interferences can be decreased by adding some solution which includes a specific chemical, like lanthanum or strontium, into the sample. The solution will work as a releasing agent in the sample. The added solution will protect the analyte by reacting with the interference substances when the analyte can be completely atomised. Additionally, a higher flame temperature and a fuel-rich flame usually decrease chemical interferences, if they just can be used in the analysis. (Harris 2007.) The interfering anions may also be totally removed from the sample by separation techniques. One other possible way to avoid chemical interferences is the addition of excess amount of the interfering anion in both the sample and standard solutions. (Lajunen & Perämäki 2004.)

6.4.2 Spectral interferences

Spectral interferences are usually caused by the overlap of the analyte's signal by the signal caused by other elements or molecules in the sample or the signal caused by the flame or furnace. By using D_2 or Zeeman background correction, the interferences caused by the flame can be eliminated. The overlap between the lines of different elements in the sample is easiest to fix by changing the wavelength of the analysis. (Harris 2007.) The other option is the removal of the interfering element from the sample. (Lajunen & Perämäki 2004). Elements that form very stable diatomic oxides cannot be defined using the flame or furnace, because the temperature is not high enough to atomise these elements completely. (Harris 2007). Spectral interferences are anyway very rarely occurred in the atomic absorption analysis. (Lajunen & Perämäki 2004).

6.4.3 Ionisation interferences

Ionisation interference can be a problem when the analyte's ionisation potential is low, because then the signal of ionised atoms will interrupt the signal of the neutral ones. This will end up decreasing the atomic signal and will cause false values of the analyte. This is especially problematic in the analysis of alkali metals at low temperatures and also with other elements at higher temperatures. One way to overcome this problem is the use of ionisation suppressor, which is a substance that will decrease the extent of the ionisation of the analyte. The ionisation suppressor is ionised more easily than the analyte, causing more analyte to stay in an atomised form in used conditions. (Harris 2007.)

6.4.4 Physical interferences

Some physical interference may also occur while measuring the atomic absorption. These interferences are caused by a change in physical characteristics of the solution to be measured, like viscosity, vapour pressure, or temperature. Physical properties are usually changed if the sample contains a large amount of salts, acids or organic compounds. That is why it is advantageous to use sample-like standards or the method

of standard addition when the sample itself is difficult to handle. (Lajunen & Perämäki 2004.)

6.4.5 Potential interfering factors in urine

In many studies, the atomic absorption technique has proved to be applicable in the analysis of many metals. Willis (1961) has proved the atomic absorption technique to be valid for calcium and magnesium determinations from urine. Gimblet etc. (1966) in turn showed that the atomic absorption technique is a simple, rapid and practical method to be used in clinical chemistry laboratories, when biological materials are under interest.

6.4.5.1 Interferences in calcium measurements

As can be discovered from the composition of urine, it contains various interference factors. These factors influence especially the determination of calcium while using the flame atomic absorption spectrometry. As Trudeau and Freier (1967) discovered, the atomic absorption spectrometry of calcium is disturbed by the presence of proteins, cations and anions, which will form complexes with calcium. Willis (1961) in turn showed that the presence of phosphorus is a significant factor in causing chemical interferences in the AAS measurements of urine.

Trudeau and Freier (1967) mentioned sodium, phosphate, sulphate, citrate, and oxalate as possible interference factors for calcium determinations. They mentioned that phosphate can cause the depression of calcium absorption. This interference can however be overcome with the addition of chemically similar metals to calcium, such as lanthanum or strontium. The overall result from Trudeau and Freier's (1961) study was that in urine analysis the interferences in calcium measurements are eliminated by adding 0.5% of lanthanum to the samples and standards.

Willis (1961) reported that 10,000 ppm of strontium or lanthanum are the most effective suppressors of the interference at high phosphorus concentrations in urine. These results were obtained in direct dilutions as well as in measurements conducted after the calcium was first separated by the precipitation with oxalate.

6.4.5.2 Interferences in magnesium measurements

In the article from Gimlet etc. (1966), the diluents of 0.25% strontium chloride and pure water were tested in magnesium measurements using the atomic absorption spectrophotometer. The results from these studies showed that water as diluent gives as good recovery as the strontium chloride diluent. The results from these measurements were close to 100% of recovery. Based on the introduced results, water was preferred as diluent when magnesium is determined because of its simplicity.

In the article by Willis (1961), the results for recovery of magnesium with water as diluent were close to 100% also. This indicates that water as diluent should be valid and the presence of other substances in the urine should not cause any interference in the analysis. In Willis' studies, an air-acetylene flame and a hollow cathode lamp were used in the performed measurements.

8 VALIDATION

8.1 The process of validation

The process of validation is performed to prove that the available method for analysis is convenient to use as it is intended. In the method, the parameters of the performance are tested by a series of planned measurements, the extent of which is chosen at the beginning of the validation process. The main aim is to indicate the reliability of the method, which is confirmed by obtaining the arranged results. The method has to be changed or improved if the arranged results are not received. (Lehtonen & Sihvonen 2006.)

The validation is usually performed when a totally new method is created or the used method is widely improved or changed. When a new laboratory starts to use an already validated procedure, it should carry out its own validation. The validation should also be performed when a new instrument is introduced. The extent of the validation depends on the use of the method, the equipment, the environment, and the personnel. In some cases, public officer demands have to be taken into consideration too while performing the validation process. (Ehder 2005.)

The sample quality has to be taken into consideration while designing the validation process. Every sample has always one or several examinee subjects and some matrix that can affect the concentration of the measured analyte. The analysis can be totally specific, if the measured signal comes only from the analyte under examination. (Jaarinen & Niiranen 2005.)

The validation process begins by creating the plan for experiments to be carried out in a given situation. After that, the measurements are performed, and the results are calculated based on them. At the end of the validation process, the instructions of the method are created and the procedure of quality control is designed. The instructions of the method include for example information about the basic principle, application tract, instruments and reagents to be used, as well as the instructions on how to calculate the results. Many different parameters can be studied in the validation process. These parameters can be for example repeatability of the method, linearity, the yield, and the limits of detection and quantitation. There is a wide selection of other parameters also,

but this study focuses only on the previously introduced parameters. (Jaarinen & Niiranen 2005.)

8.2 Mean and standard deviation

In the laboratory, the measurements are usually performed as parallel and the mean of the measurements is then calculated from the results. The formula of mean is shown in formula 3 (Lehtonen & Sihvonen 2006).

$$\bar{x} = \frac{x_1 + x_2 + x_3 + \dots}{n}, \text{ where the} \quad (3)$$

$x_1/x_2/x_3 \dots$ = measured values

n = the number of measurements

The standard deviation describes the dispersion of the measurements around the mean value. The standard deviation of the individual measurement describes also the precision of the measurements. Standard deviation is calculated as shown in formula 4 (Lehtonen & Sihvonen 2006.)

$$S = \sqrt{\frac{(x_1 - \bar{x})^2 + (x_2 - \bar{x})^2 + (x_3 - \bar{x})^2 + \dots}{n - 1}}, \text{ where the} \quad (4)$$

$x_1/x_2/x_3 \dots$ = measured values

\bar{x} = mean

8.3 The repeatability of the method

Repeatability is the standard deviation of the same sample divided into small parts, which are measured in similar conditions during a short period of time. While calculating repeatability, the personnel, instrument and the reagents should all be the same at all times. (Jaarinen & Niiranen 2005.)

The repeatability accuracy is achieved from measurements of the same sample and its parallel samples. For the aim of the accuracy, some value, like $RSD < 5\%$, can be used for deviation or relative deviation. Typically, the RSD in the lower point of measuring range is 3–30%, because the accuracy of repletion is the function of the concentration and will increase while the concentration decreases. The relative standard deviation is calculated in formula 5. (Lehtonen & Sihvonen 2006.)

$$(\%)RSD = \frac{st.dev.}{mean} \times 100\% \quad (5)$$

8.4 Linear working range

In the linear measuring range, the sensitivity of the instrument is constant, which means that there is a linear correlation between the results and the concentrations of the analysed analyte in the samples. In this working range, the error of the instrument is within the known and specified limits. In linear working range, the level of measured signal should be directly proportionate to the concentration of the sample. At the same time, the calibration curve has to be linear. (Jaarinen & Niiranen 2005.)

The linear working range in quantitative analysis should always be used when possible. Then the changes in concentration will cause the same size difference to the signal, and the sensitivity will be constant in the entire area of measurements. The sensitivity is the same value as the value of the slope in the calibration curve. The sample should be diluted if its concentration is outside the linear area. (Jaarinen & Niiranen 2005.) Measurements should be carried out in the area where there is a linear connection between the measured variable and the variable that the equipment gives. In this situation, the calibration graph is straight and the sensitivity is constant in the whole measuring range. Formula 6 shows how sensitivity is calculated. (Lehtonen & Sihvonen 2006.)

$$sensitivity = \frac{signal}{concentration} \quad (6)$$

8.5 The reliability of calibration

The reliability of calibration can be estimated with a correlation coefficient, which can vary between the values -1...+1. When the value is very far from number 1, calibration measurements can be expected to contain either dispersion or nonlinearity. In an ideal situation, the square of the correlation coefficient is 1. The linearity should never be estimated only with this value. (Jaarinen & Niiranen 2005.)

The graphical picture of residual plots is a good factor to be used with the correlation coefficient while estimating the linearity of the standard curve. Residual is the difference between measured calibration plots and the precalculated calibration plots. Linearity can be obtained from the graphical picture when the plots are spread evenly. (Jaarinen & Niiranen 2005.)

8.6 The yield

Yield is the effectiveness of the method in observing the absolute concentrations of the examined analyte. Many factors are always present in the chemical pretreatment of the defined analyte. Some of these factors may even be difficult to identify. That is the main reason to determinate the yield of the analyte in every new method. One way, which is used in this study also, is to study the yield with the known additions of the pure analyte. (Ehder 2005.) The yield is usually reported as the percentage of the calculated value of the known addition. The calculation is presented in formula 7.

$$(\%)R = \left[\frac{c_1 - c_2}{c_3} \right] * 100, \text{ where the} \quad (7)$$

c_1 =the average of measured concentration with a known addition

c_2 =the average of measured concentration of the sample without an addition

c_3 =the calculated concentration of the addition

8.7 Limits of detection and quantitation

The limit of detection is the smallest concentration of the defined analyte, which can be reliably specified and which differs remarkably from the zero-sample. (Ehder 2005). The limit of detection is calculated in formula 8.

$$x_{LOD} = \frac{3 \times S_{bo}}{b_1}, \text{ where the} \quad (8)$$

S_{bo} =standard deviation of the intersection of the standard curves

b_1 =the slope of the standard curve

The limit of quantitation is the smallest amount of the analyte with which the quantitative measurements can be performed in a reliable grade. (Ehder 2005). The limit of quantitation is calculated in formula 9.

$$x_{LOQ} = \frac{10 \times S_{bo}}{b_1}, \text{ where the} \quad (9)$$

S_{bo} =standard deviation of the intersection of the standard curves

b_1 =the slope of the standard curve

9 MATERIALS AND METHODS

9.1 The sample

Real urine was decided to be used in this study, because the complexity of the urine matrix made it very difficult to prepare reliable synthetic urine. Urine was collected from voluntary co-workers and members of their families in the age range between 2 to 50 years. Both male and female were included. The samples were collected in 400 millilitre plastic cups with covers. Fresh urine samples were always stored in the refrigerator (+4°C) for a maximum of a couple of days before the studies started.

The urine samples used in the studies were always mixed urine samples from 2 to 6 donators. The samples were combined in 2 or 5 litre volumetric flasks and mixed well. From the volumetric flask, the mixed urine was divided into sample plastic cups with covers, which can be seen in picture 2. Each sample contained 150 millilitres of combined urine, which was measured with a measuring glass. Between dividing the sample to its replicates, the volumetric flask was mixed carefully.



PICTURE 2. Urine samples in the heated cabinet. (Picture taken by Mäkinen, H. 2011)

A certain amount of specific substance was added to some samples on the first day of the study. Different reactions in the ureolysis process were expected to take place in these samples. The effectiveness of these substances was tested by analysing the concentration of soluble metals magnesium and calcium at the beginning and at the end of the study. The soluble amount of these metals in the sample's liquid phase is an important factor when predicting the occurrence of precipitates. These studies were however classified information, so the results are not introduced here.

The samples were kept in a heated cabinet between daily or weekly performed measurements. The cabinet was adjusted to +20 °C to maintain a similar atmosphere to all the samples at all times. Periodically performed measurements included pH, ammonia and conductivity measurements.

From the fresh combined urine, three parallel samples were taken in the starting point for the analysis of metals. These samples were the control ones of the sequence. On the first day, the initial pH and conductivity were measured from each sample. On the second day, the pH, conductivity, and ammonia were measured from each sample which was included in the series of samples. After the second day, these measurements were carried out to each sample at least once a week until the pH of the control samples reached the pH value of 8.5–9, which took approximately one month.

The ureolysis period was completely ready when the pH value of over 8.5 was reached. In this point, the studied metals calcium and magnesium were spent almost as far as possible. This fact was also indicated in various articles, for example in Udert (2003). When this endpoint pH was reached in the control and its parallel samples, the samples for analysis were collected from every sample in that sequence, regardless of the pH value in the individual samples. The samples were taken into 15 ml plastic tubes and frozen until the AAS measurements were performed. Urine samples with low pH were kept in the heated cabinet to continue the study and research the long term effects of the added substances.

9.2 Measurement of study

9.2.1 pH measurement

The pH was measured at the beginning and at the end of the study, and also at least once a week during the ureolysis process. The pH was measured with the pH meter ORION model SA 720 + ORION 8172BNWP ROSS Sure-Flow Combination pH electrode.

The meter was calibrated daily using two-point calibration with the calibration solutions of 2, 7 and 10, which were commercial products. After the calibration was performed, the meter was ready for use and the samples were measured. Between every sample and control, the meter was rinsed with milliQ water and dried well with a fine paper.

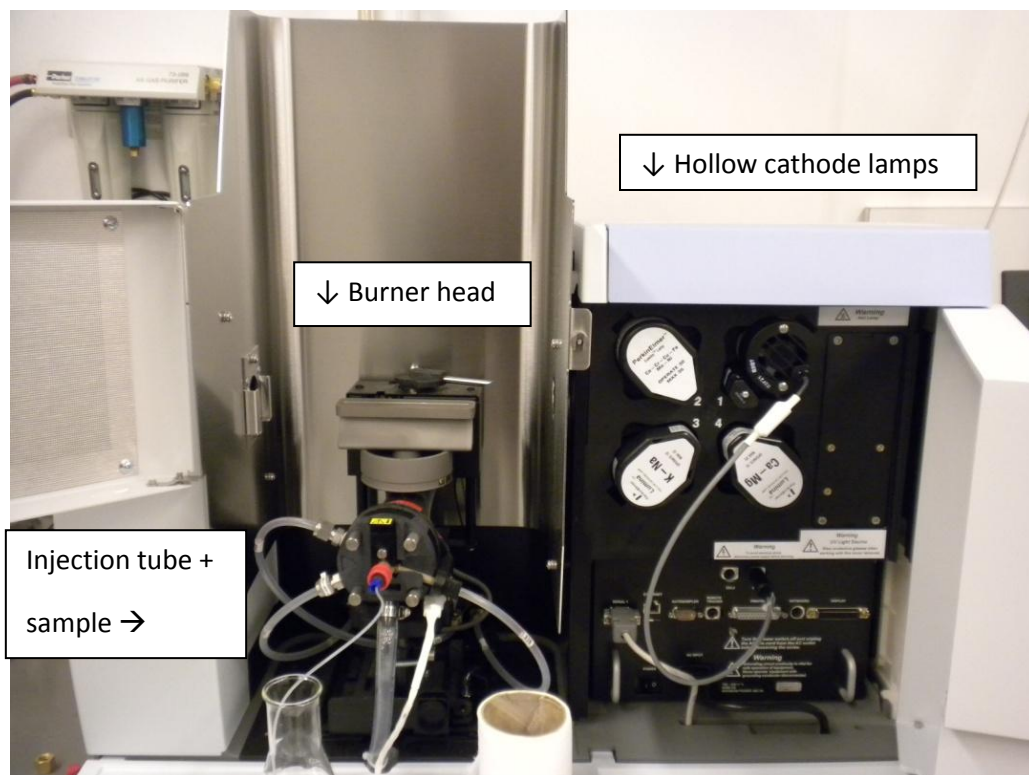
9.3 The AAS measurements

The flasks which were used in the preparation of the AAS samples were acid washed, as recommended in the standard SFS 3044, to make sure that all the distracting materials were removed from the surfaces of the glassware. For the acid washing procedure, the volumetric flasks were first filled up with EDTA solution which contains 5% of detergent and 1% Na-EDTA in a one litre solution. The EDTA solution was left in the volumetric flasks for overnight and then the bottles were rinsed with milliQ water three times. Then the bottles were filled up with washing acid, which contains 7 ml of nitric acid (65%, analytical grade from MERCK) in one litre of the solution made with milliQ water. The duration of action with washing acid was at least two hours, after which the bottles were rinsed with milliQ water three times and placed upside down in a clean place to dry before the use.

All samples were filtrated before the analysis. For the filtration procedure, the 25 mm syringe filters from VWR international (0.45 μ m Nylon) were used. First, they were washed three times with milliQ water by pushing the water through the filter with a 10 ml Sterile BD Discardit syringe, and after the washing cycle the filters were emptied from water by pulling the syringe against the vacuum to get all the water drops out of the filter. When the actual sample was filtrated through the cleaned filter, the first drops were wasted and the rest of the sample was collected into a clean acid washed measuring glass. The samples which were taken after the ureolysis procedure needed on

average four filters because of the blockage caused by the precipitated subjects, while the fresh samples went easily through only one filter. The filtered samples were then diluted and otherwise prepared for the AAS analysis.

The measurements were performed with flame atomic absorption spectrometry using PerkinElmer, the Aanalyst 400 Atomic absorption Spectrometer. The used lamp was multielement Lumina™ Hollow cathode lamp Mg-Ca from PerkinElmer. The air-acetylene gas was used as a carrier gas in both methods. In magnesium measurements, the used wavelength was 285.2 nm with the background corrector on and in calcium measurements the wavelength was 422.7 nm. The slit was not changed from the one that the AAS equipment gave for the chosen wavelength. The AAS equipment used is shown in picture 3.



PICTURE 3. The AAS equipment from inside picture by Mäkinen H. 2011

9.3.1 Starting method

The analysis of magnesium and calcium were started by the Drycloset group by following the standard methods SFS 3018 and 3044. The standard stock solutions were bought from MERCK and they included 1000mg/l of the analysed metals. The LaCl_3 solution, used to minimise the distraction effect, was prepared in the laboratory by following the recipe in standard SFS 3018. The amount of LaCl_3 solution added into the calcium samples and standards was 1 ml per 100 ml of the sample. The calibration curve was drawn using only one standard concentration. This point was chosen to be the highest concentration in the linear working range.

The sample dilutions for the measurements of fresh urine were 1:200 for the magnesium analysis and 1:100 for the calcium analysis. The urine samples were always filtered through 0.45 μm filters before dilutions. All samples and standards were prepared in acid washed glassware, washing procedure described in standard SFS 3044.

9.3.2 First improvements of the method

The initial method was slightly modified already before any particular tests for the method were actually performed. First, the standard curve was decided to be prepared using three different standard concentrations instead of only one standard concentration. Different dilution factors for the samples were tested and new ones found to be used as introduced in table 2.

TABLE 2. Preparation of the samples

Sample	Dilution factor	Pipette amount of sample / size of volumetric flask
Fresh Mg	1:400	250 μl /100ml
Fresh Mg	1:250	400 μl /100ml
Stored Mg	1:100	1 ml/100ml
Stored Mg	1:20	2,5 ml/50 ml
Fresh Ca	1:50	1 ml/50ml
Stored Ca	1:20	2.5 ml/50 ml

At first, the test for calcium analysis was performed. The samples were diluted in milliQ water and 0.1% of LaCl_3 solution was added into the standards and samples. The results of these tests did not reach satisfying limits. Following these unsatisfactory results, a deeper literature survey was performed. This survey indicated that the determination of calcium from urine needed at least 0.5% addition of lanthanum or strontium to overcome the interferences in urine. This fact was pointed out in the article by Trudeau & Freyer (1967).

Many of the surveyed studies also showed that the recovery of magnesium was reached nearly 100% while only water was used as diluent. In our studies, the recovery with milliQ water as a diluent did not reach satisfying limits, which was set to $100 \pm 5\%$. After all these results, more testing was decided to be performed for both of the methods.

9.3.3 Final improvements of the method

The LaCl_3 solution prepared in the laboratory was changed into a commercial one. The LaCl_3 solution had run out of stock, so the $\text{CsCl} + \text{LaCl}_3$ solution was used to replace that. The $\text{CsCl} + \text{LaCl}_3$ solution was proved to be suitable for the calcium and magnesium analyses in the AAS measurements by Schinkel (1984). The used solution was cesium chloride-lanthanum chloride buffer solution according to Schinkel for atomic absorption spectroscopy included enth./cont. 10 g/l CsCl + 100 g/l La and it was produced by MERCK.

Based on the results from the literature, the added amount of $\text{CsCl} + \text{LaCl}_3$ was increased from 0.1% to 0.5% volume in the calcium analysis. Different environments, milliQ water versus acids HCl and HNO_3 , were also subjected to tests. The results from these tests were mainly compared based on the amount of recovery, which was decided to be the most important factor in the measurements. The used acids were 1 M HNO_3 and 1 M HCl and their amounts were designed so that the sample's pH went under pH 2. In some articles, the urine was preserved in hydrochloric acid before the analysis, for example in Willis' (1961) studies. In our studies, this kind of sample preserving was not performed.

The magnesium tests were redone in the presence of 0.05% of $\text{CsCl} + \text{LaCl}_3$ solution in the sample volume, even though according to the literature, the results should have been good without any additions. The matrix of the real urine is so complex that the addition

of $\text{CsCl}+\text{LaCl}_3$ was decided to be tested in this study to see whether it affects the recovery or not.

9.4 Validation factors

9.4.1 Mean, standard deviation and RSD

The standard 3, which is located in the middle of the standard series, was aspirated six times for the use of the first validation tests. This standard was prepared in a similar way as the samples, so the same addition of $\text{CsCl}+\text{LaCl}_3$ was included in it. From the received measurement values, the mean and standard deviation (S) of the absorbance were calculated. The formulas to calculate these factors are shown in formulas 3 and 4. From these values the relative standard deviation (RSD) as percentage was calculated using formula 5, and repeatability was evaluated based on this result.

9.4.2 Linearity and working range

The linearity and working range of the method were defined by aspirating the six standard solutions with different concentrations three times each. The concentrations are shown in tables 3 and 4. The highest concentration of the standard curve was selected based on the linearity range showed in recommended conditions (The Perkin-Elmer Corporation 2006). These results were used when the linearity was defined by checking the residual plots and analysing the linearity from graphic.

TABLE 3. Standard concentrations for calcium standard curve

Standard number	Concentration (mg/l)
1	0.5
2	1.0
3	2.0
4	3.0
5	4.0
6	5.0

TABLE 4. Standard concentrations for magnesium standard curve

Standard number	Concentration (mg/l)
1	0.05
2	0.1
3	0.25
4	0.3
5	0.4
6	0.5

9.4.3 Repeatability of method

The repeatability of the method was tested by making the same dilution of the sample solution six times, so that six replicates were achieved. Each of these samples was aspirated once and the amount of the defined analyte (Ca or Mg) in the sample solution was calculated by taking note of the dilution factor. The instructions for preparing the correct sample are introduced in table 2. From the measured values, the mean and standard deviation of the absorbance were calculated using formulas 3 and 4. Then the relative standard deviation (RSD) and the system's repeatability were calculated and evaluated using the formula 5.

9.4.4 Accuracy and recovery

For accuracy and recovery test, four samples with the same dilution were prepared and three of these similarly prepared samples were spiked with examinee standard solution (Mg or Ca) with three different concentrations. The spiked concentrations of magnesium stock solution were 0.05, 0.1 and 0.2 mg/l, which were pipetted from the working solution (100mg/l). The working solution was always prepared from a commercial stock solution on the day of analysis. Calcium was also spiked by using the working solution (100mg/l), which was prepared from the commercial stock solution as well. The spiked concentrations of calcium were 0.5, 1.0 and 1.5 mg/l. All these

samples were aspirated three times each and the mean recovery for each sample was calculated using formula 7.

9.4.5 Limits of detection and quantitation

The limits of detection and quantitation were defined from the calibration curves, which were drawn based on the three different aspirations of the six standard solutions with different concentrations, shown in tables 3 and 4. The standard curves were drawn with Microsoft Excel software and the limits were calculated using formulas 8 and 9. Some of these standard curves are shown in pictures 4 and 6 and the correlations of every standard curve are introduced in the results.

10 RESULTS

In all the following results, the stored urine means the urine which has been measured after it has gone through the ureolysis process. The stored samples used in the AAS tests were always control urine, so they were not treated with any substances. Only the pH, ammonia levels, and conductivity were followed and the samples were frozen or analysed when the pH reached the value between 8.5 and 9 and the precipitation was known to be nearly completed.

10.1 Magnesium methods

The correlations of the standard curves were examined from each series. The results are shown in table 5. The results were calculated for both the initial and improved methods using the fresh urine and for the improved method using the stored urine too. Table 5 introduces also the results for the detection and quantitation limits, which were calculated by using formulas 8 and 9.

TABLE 5. Results of correlation and limits of detection and quantitation for Mg-methods

	Mg-old protocol 1.9.2011	Mg-new protocol 20.10.2011	Mg-new protocol 20.10.2011 (stored)	Mg-new protocol 29.9.2011
Correlation in standard curve	0.998	0.999	0.999	0.999
Limit of detection	0.00078	0.00105	0.00105	0.037 *
Limit of quantitation	0.0026	0.0035	0.0035	0.0071 *

* The standard curve is determinate only with 3 different standard concentrations.

To evaluate the system's repeatability, the mean, standard deviation and relative standard deviation of the absorbance were calculated from the measured results with formulas 3, 4 and 5. The obtained results were slightly lower in the measurements of the

initial method than the results from the improved method. The difference in these results was however very small. The results are shown in table 6.

TABLE 6. System's repeatability in magnesium measurements

	Mg-old protocol 1.9.2011	Mg-new protocol 20.10.2011	Mg-new protocol 20.10.2011 (stored)
Mean of the measured absorbance	0.31	0.36	0.36
Standard deviation	0.00055	0.00084	0.00084
(%)RSD	0.18	0.23	0.23

To determine the repeatability of the method, the mean, standard deviation and the relative standard deviation were defined from the results by using formulas 3, 4 and 5. The calculations were performed from the results of final concentrations of the samples and they are presented in table 7. The measurements were performed with the initial and improved method by using fresh urine and also with the improved method by using the stored urine.

TABLE 7. Method repeatability in magnesium determinations

	Mg-old protocol 1.9.2011	Mg-new protocol 20.10.2011	Mg-new protocol 20.10.2011 (stored)	Mg-new protocol 29.9.2011
Mean concentration of samples (mg/l)	59.8	68.4	0.17	124.7
Standard deviation	0.83	0.64	0.01	0.69
(%)RSD	1.39	1.69	5.96	0.56

For recovery tests, the similar dilutions of the sample were prepared four times and three of these samples were spiked with different amounts of standard solution as explained in the materials and methods section. The tests were performed with initial and improved methods and the results calculated with formula 7 are presented in table 8. The results which were not on a satisfying level are highlighted with red colour.

TABLE 8. The recovery results for magnesium methods

	Mg-old protocol 1.9.2011	Mg-new protocol 20.10.2011	Mg-new protocol 20.10.2011 (stored)	Mg-new protocol 29.9.2011
Correlation in standard curve	0.998	0.999	0.999	0.999
Recovery 1 (%)	84	110	86	92
Recovery 2 (%)	80	106	100	98
Recovery 3 (%)	80,5	103	104	95.5

10.2 Calcium methods

The correlations of standard curves in calcium measurements were on a good level as shown in table 9. The results were calculated for both the initial and improved methods. From these measurements, also the results for detection and quantitation limits were defined with formulas 8 and 9 and the results are shown in table 9.

TABLE 9. Results of correlation and limits of detection and quantitation for Ca-methods

	Ca-old protocol 8.9.2011	Ca-new protocol 13.10.2011	Ca-new protocol 18.10.2011	Ca-new protocol 18.10.2011 (stored)
Correlation in standard curve	0.999	0.999	0.999	0.999
Limit of detection	0.037	0.061*	0.028	0.028
Limit of quantitation	0.123	0.202*	0.008	0.008

* The standard curve is defined only with 3 different standard concentrations.

To evaluate the system's repeatability, the mean, standard deviation and relative standard deviation of the absorbance were calculated from the measured results by using formulas 3, 4 and 5. The results were calculated for the initial and improved method by using the fresh urine and for the improved method by using the stored urine. These results are presented in table 10.

TABLE 10. System's repeatability in calcium determinations

	Ca-old protocol 8.9.2011	Ca-new protocol 18.10.2011	Ca-new protocol 18.10.2011 (stored)
Mean of the measured absorbance	0.09	0.17	0.17
Standard deviation	0.0006	0.0010	0.0010
(%)RSD	0.70	0.62	0.62

The mean, standard deviation and relative standard deviation of the samples' concentrations were defined to evaluate the repeatability of the method by using formulas 3, 4 and 5. The calculations were made for the initial and improved method by using the fresh urine and for the improved method by using stored urine. The obtained results are shown in table 11.

TABLE 11. Method repeatability in calcium determinations

	Ca-old protocol 8.9.2011	Ca- new protocol 13.10.2011	Ca- new protocol 18.10.2011	Ca- new protocol 18.10.2011 (stored)
Mean concentration of samples (mg/l)	55.4	124.15	74.3	1.9
Standard deviation	0.599	0.606	0.375	0.267
(%)RSD	1.08	0.49	0.50	14.42

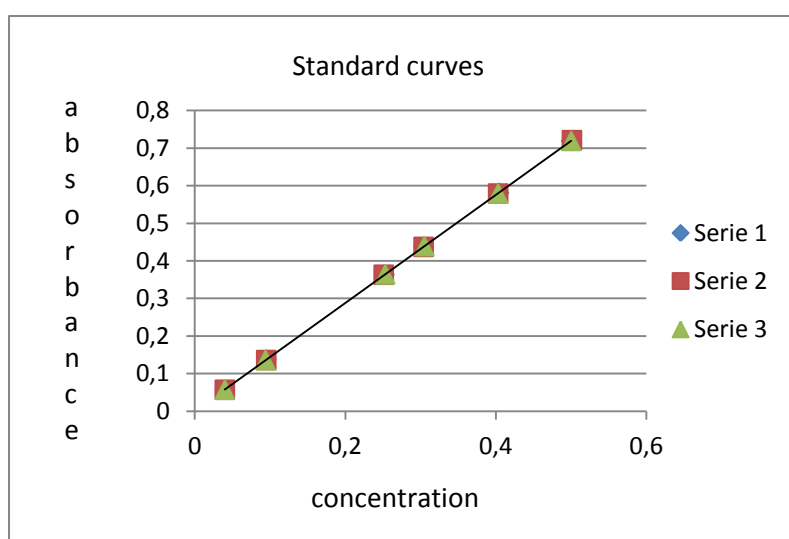
The recovery as percentage was defined for both the initial and improved calcium methods using formula 7. The results are shown in table 12 where the red markings represent the results which did not reach the defined level of satisfaction.

TABLE 12. The recovery results for calcium methods

	Ca-old protocol 8.9.2011	Ca-new protocol 13.10.2011	Ca-new protocol 18.10.2011	Ca-new protocol 18.10.2011 (stored)
Correlation in standard curve	0.999	0.999	0.999	0.999
Recovery 1 (%)	80.9	101.7	89	100.2
Recovery 2 (%)	80	102.6	98.2	98.2
Recovery 3 (%)	79.7	102.6	97.1	101.3

10.3 Linearity in magnesium method

A standard series with six different concentrations were prepared and measured three times. The standard curves were drawn from the obtained results. The curves for improved magnesium method are shown in picture 4.



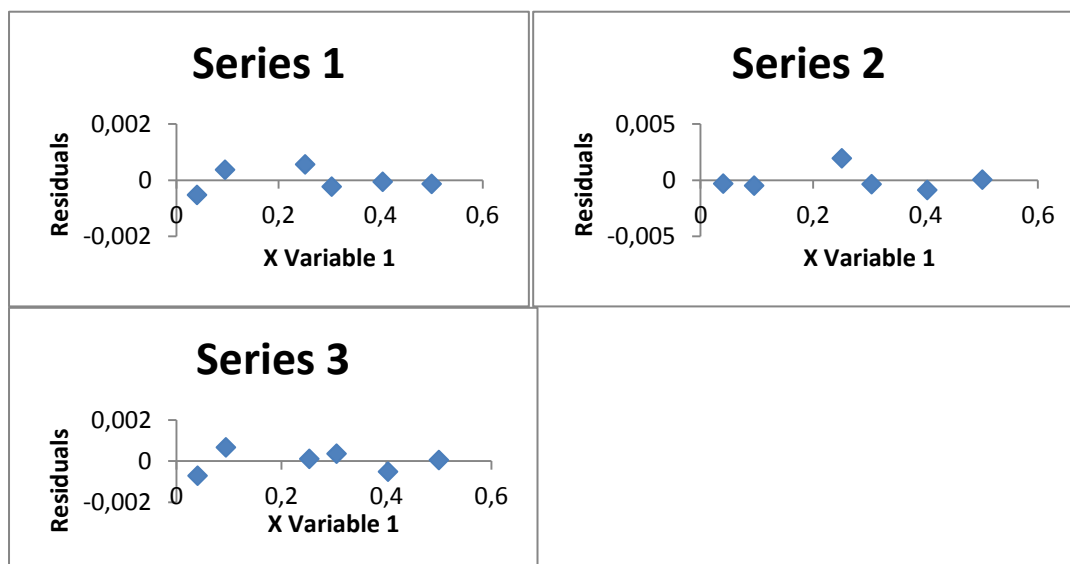
PICTURE 4. The standard curves of magnesium measurement on 20.10.2011

The square of correlation coefficient and the equation of the curve for standard curves were defined with Microsoft Excel software. The results for the improved magnesium method are presented in table 13.

TABLE 13. The correlation and equation of the magnesium measurement on 20.10.2011

Series	Equation of the curve	r^2 , the square of correlation coefficient (calculated with MS Excel software)
1	$y=1.4383x-8E^{-6}$	1
2	$y=1.4396x-0.0003$	1
3	$y=1.4375+0.0002$	1

To study the linearity of the methods, the residual plots were defined and drawn. The residual plots for the improved method of the magnesium are shown in picture 5 and the plots are introduced as numbers in appendix 2.



PICTURE 5. Residual plots magnesium 20.10.2011

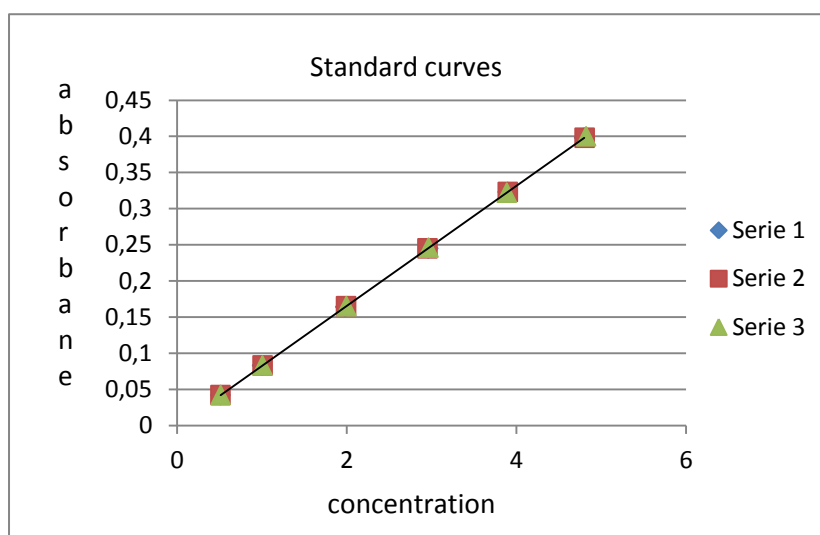
10.4 Linearity in calcium method

The square of correlation coefficient and equation of the curve for standard curves were defined using Microsoft Excel software. The results for the improved calcium method are represented in table 14.

TABLE 14. The correlation and equation of the calcium measurement on 18.10.2011

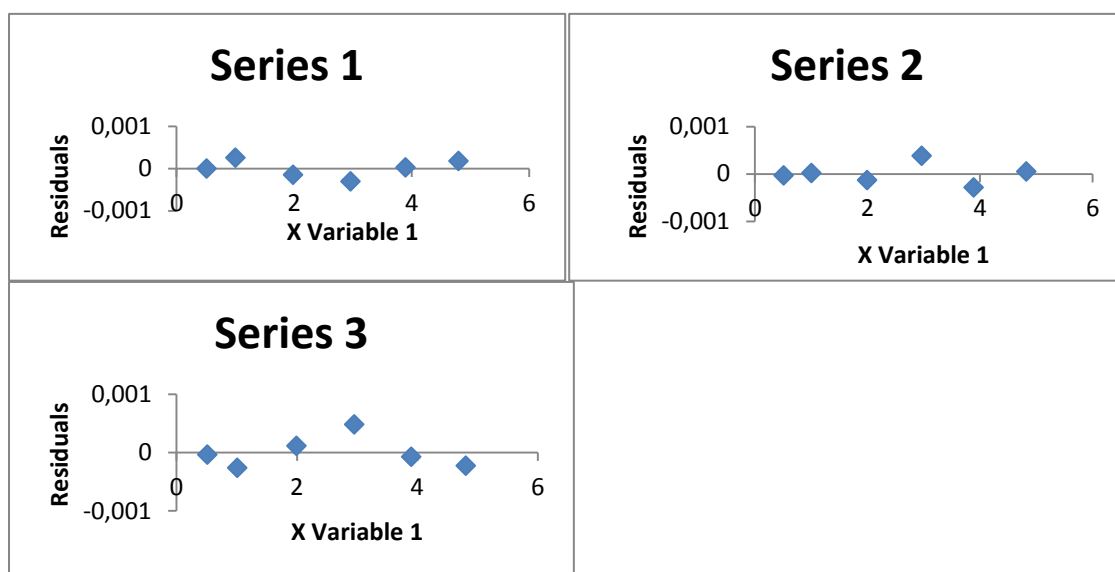
Series	Equation of the curve	r^2 , the square of correlation coefficient
1	$y=0.083x-0.0003$	1
2	$y=0.083x-0.0003$	1
3	$y=0.0831x-0.0005$	1

The linearity of the improved calcium methods was illustrated by drawing the standard curves. The curves shown in picture 6 are from three parallel standard curves.



PICTURE 6. The standard curves of calcium measurement on 18.10.2011

The graphs of residuals for the improved calcium method are shown in picture 7. The plots are also introduced as numbers in appendix 2



PICTURE 7. Residual plots Ca-new protocol 18.10.2011

10.5 Acid tests

The improved method of calcium was tested in different environments which were milliQ water, HCl, and HNO₃. The results of the recovery tests are introduced in table 15 where the red marking is used to indicate the unsatisfying results.

TABLE 15. The recovery results from environmental testing

	HCl	HNO ₃	milliQ-water
Correlation in standard curve	0.999	0.999	0.999
Recovery 1 (%)	101.7	97.2	110.3
Recovery 2 (%)	102.6	96.9	99.3
Recovery 3 (%)	102.6	100.3	101.3

The repeatability of the method in different environments was also studied by determining the mean, standard deviation, and relative standard deviation with formulas 3, 4 and 5. The results obtained with the improved calcium method are presented in table 16.

TABLE 16. Results in the method's repeatability in environmental testing

	HCl	HNO ₃	milliQ-water
Correlation in standard curve	0.999	0.999	0.999
Mean concentration of Ca-samples (mg/l)	123.5	119.6	121.8
Standard deviation	0.520	0.361	0.898
(%)RSD	0.42	0.30	0.74

10.6 LaCl₃ solution as diluent

Some comparative tests were performed with improved calcium and magnesium methods by using LaCl₃ solution as diluent instead of CsCl+LaCl₃ solution. The results of the standard curve's correlation and of the limits of detection and quantitation are shown in table 17.

TABLE 17. The correlation and limits of detection and quantitation measurements with LaCl₃ solution

	Mg 17.10.2011 LaCl ₃	Ca 17.10.2011 LaCl ₃
Correlation in standard curve	0.999	0.999
Limit of detection	0.0010	0.018
Limit of quantitation	0.0033	0.06

The repeatability of the method was also studied for both magnesium and calcium improved methods. To study the repeatability, the mean, standard deviation and relative standard deviation of the sample concentrations were defined. The results are introduced in table 18.

TABLE 18. Method repeatability in measurements with LaCl_3 -solution

	Mg 17.10.2011 LaCl_3	Ca 17.10.2011 LaCl_3
Mean concentration of samples (mg/l)	121.6	199.2
Standard deviation	0.327	1.374
(%)RSD	0.27	0.69

Recovery results for magnesium and calcium initial methods using LaCl_3 solution as diluent are shown in table 19. The unsatisfying results are marked with the red colour.

TABLE 19. The results of recovery in measurements with LaCl_3 solution

	Mg 17.10.2011 LaCl_3	Ca 17.10.2011 LaCl_3
Correlation in standard curve	0.999	0.999
Recovery 1 (%)	110.7	109.5
Recovery 2 (%)	104.7	101.2
Recovery 3 (%)	103.7	Result discarded, because its concentration higher than standard line

11 OVERVIEW OF THE RESULTS

The atomic absorption measurements of calcium and magnesium were done to investigate how much of these metals were in a liquid phase in fresh and stored urine. The difference in their amounts in fresh urine at the starting point versus at the end point in stored urine is equivalent to the level of precipitations occurred during the storing time. The AAS equipment was not familiar for all participants in the research group, and the performance of the created method was not previously tested, so the method was decided to be tested with some validation factors, of which the yield was the most important one. Based on the results of these tests, some modifications for the method were performed.

At the very beginning, only one standard concentration for the measurements of the standard curve was in use, and it was directly changed to a minimum of three standards per measurement. The changes were done because the use of one standard is only suitable while checking the settings of equipment, but not in actual analysis, as Jaarinen and Niiranen (2005) have stated. Some modifications for the dilution factors, which were initially taken from the AAS user guide (Perkin-Elmer corporation, 2006), were also performed to obtain the measuring results approximately in the middle of the standard curve, which is usually the most accurate place to estimate the results.

The linearity of the calibration for magnesium and calcium measurements was defined with the improved methods. With both metals, the correlation coefficient was the best possible in all three measured standard series, which indicates a good linearity. The correlation coefficient obtained directly from AAS equipment was slightly deviant, but being 0.999, it was still very good. The graphical pictures of the residuals confirmed the linearity in all defined standard curves, because the plots were spread quite evenly in both sides of the line, which represents a good linearity. Following the very good and constant results from correlation and residuals, the linearity can be said to be valid in both methods.

In the first calcium and magnesium measurements with the initial method, some validation factors seemed to be on quite good and valid levels already. Such were the correlation of the standard curve, which was nearly 1, and the limits of detection and quantitation which were on relatively good levels. Although the results for the limits of detection and quantitation seemed quite good with the initial method, the results later

revealed that especially the limit of quantitation improved quite much with the improvements. A good correlation alone is not a sufficient factor to evaluate the quality of the method, and other factors, such as recovery, proved further that some modifications for the methods were needed.

The main problem in the initial calcium method was the yield, because the recovery percent was only about 80% and even though the matrix of urine is very complicated, the results were not on a satisfying level, which was defined to be $100\pm 5\%$ in this study. A literature survey showed also that the recovery of nearly 100% is possible to reach with both studied metals in urine samples as presented in the results of Willis' (1961) studies. The extended literature survey revealed that the amount of used LaCl_3 solution or later $\text{CsCl}+\text{LaCl}_3$ solution needed to be increased into higher levels to overcome all the interferences in urine samples.

The amount of $\text{CsCl}+\text{LaCl}_3$ solution was increased from 0.1% level up to the level of 0.5% in the samples for calcium measurements. The decision to test this new amount was based on the literature. Like in Trudeau & Freier's (1967) studies, this amount was discovered to be the smallest one to eliminate the interfering effects of urine matrix. With this improvement, the recovery in calcium measurements increased into satisfying levels $100\pm 5\%$, and at the same time all other validation factors were on valid levels too.

The system's repeatability in the calcium measurements stayed in quite similar values after the modifications were executed, which proves that the equipment was working evenly all the time. On the other hand, the repeatability of the method improved somewhat with the improvements made to the protocol. The (%)RSD value lowered from 1.08 to a level around 0.5, which is already a notable improvement. Even though the standard deviation was really good in all the measurements after the modifications, the (%)RSD value obtained with stored urine was as high as 14.42, which indicates a poor repeatability. The most likely explanation for this unsatisfactory result is the very low concentration of calcium measured in the stored samples. This causes the results to stay in the area of low values, and as Jaarinen & Niiranen (2005) point out, it may increase the (%)RSD values to even as high level as 30%, which causes the results to be unreliable and incorrect.

The literature survey revealed that the recovery of magnesium while measuring the urine samples reached the level of 100% when only milliQ water was used as eluent, as

was reported in Willis' (1961) article. In our studies, the recovery of magnesium with milliQ water as eluent reached only the level of 80% and it was not a satisfying result for us, because it did not reach the required level which was $100\pm 5\%$. Approximately the same level was reached also while HNO_3 or HCl were used as eluent, but these results are not reported in this paper. Therefore, it was decided that some amount of $\text{CsCl}+\text{LaCl}_3$ solution was to be added into the magnesium samples to prevent possible interferences caused by the complex matrix of urine. The results prove clearly that the addition of 0.05% $\text{CsCl}+\text{LaCl}_3$ solution already increased the recovery percent in magnesium measurements into the new level of $100\pm 8\%$, which was almost in the required satisfying level and it was decided to be good enough for our studies.

The modifications of magnesium method did not improve the other defined factors significantly. The detection and quantitation limits were very similar in the measurements performed with the initial and improved methods. In the initial protocol, these values were actually even slightly better. The exactly same effect can be seen in the results of the system's repeatability.

In the repeatability of the method, the results' (%)RSD values varied a little in the measurements made on different days. No typical trend between the initial and improved method could be seen however. A similar effect as with the calcium measurements can be seen in the results of stored urine, when the (%)RSD value is little above 5%. The same explanation as with calcium fits in this situation too, because the measured concentration in the stored magnesium sample was only 0.17 mg/l.

The solution of $\text{CsCl}+\text{LaCl}_3$ was not previously tested in the urine analysis, but its suitability for AAS analysis in both magnesium and calcium measurements were reported by Schinkel (1984). In our studies, the $\text{CsCl}+\text{LaCl}_3$ solution was proved to be suitable to be used in calcium and magnesium determinations from urine samples. For the comparison of efficiency, one series of magnesium and calcium measurements with LaCl_3 solution as diluent were also performed in this thesis, because its functionality has been proved in previous studies, such as Willis' (1961) study. This comparison proved that the results obtained with LaCl_3 solution were pretty similar to the results obtained with $\text{CsCl}+\text{LaCl}_3$ solution. Therefore, the conclusion was that the $\text{CsCl}+\text{LaCl}_3$ solution is suitable to be used in the AAS analysis of calcium and magnesium in urine for the purpose of diminishing the interferences caused by the matrix.

In this study, different environments as sample background were tested to find the optimal one to be used in these methods. The compared environments were milliQ water, HCl, and HNO₃. In the tests of recovery and the repeatability of the method, both acidic environments appeared to give better results than milliQ water. The repeatability in HNO₃ environment was slightly better than in HCl environment, but the difference was quite insignificant between these two. The difference between both of the acidic environments and milliQ water was more notable. In the recovery test, the HCl environment was the most stable one among all the tested environments. The other results obtained with HCl were also valid and it was thus selected to be used in the analysis of magnesium and calcium. In Willis' (1961) article, the urine samples were treated with HCl before sample preparation, which was not done in our protocol, but that supports the selection of HCl to be used as sample background.

12 DISCUSSION

This Bachelor's thesis was overall fairly successful, and the improved methods were taken into use in Drycloset group. The previous literature survey was not so widely concentrated on the AAS method. The survey proved the need for some modifications to the initial method, especially with the amounts of CsCl+LaCl₃ solution added into the samples to minimise interferences. The improvements affected significantly the factor of yield of the methods, and although no great improvements were obtained to the other validation factors, the changes to the method did not affect them negatively either. Therefore, better methods to measure magnesium and calcium levels from urine were created.

The results of this study prove that the results obtained with the improved method are reliable and can be reported forward. The interferences caused by the complex matrix of urine were greatly overcome in both improved methods with the addition of certain amount of CsCl+LaCl₃ solution into the samples and standards. Overall, the arranged limits were reached after the accomplishment of the modifications.

In the future, a more extensive validation is recommended to be accomplished, with a wider range of validation factors and more repeats, especially if the methods are taken into wider use in the Faculty of Science and Environmental Engineering at Tampere University of Technology. More testing with both acidic environments could be performed to compare them further, because these results revealed no notable difference between them.

It would be very interesting to study the forming precipitations with some other method and techniques to investigate their composition more precisely. Now the precipitation is assumed to be struvite and other precipitations. More precise information about the precipitations could provide additional resources to prevent their occurrence in pipelines. One possible technique to study the solid parts of the sample would be mass spectrometer analysis.

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APPENDIX 1

Sample preparation for determination of magnesium and calcium from urine with AAS
1(2)

Put some milliQ water into each volumetric flask prior to adding standard or sample.

Use acid washed glass bottles in all stages of the work.

Magnesium:

- Standards:

1. Make the working solution (10mg/l) daily from magnesium stock solution (1000mg/l) by pipetting 1ml of stock solution into 100 ml volumetric flask and fill up to mark with milliQ water.
2. Prepare the standards into 100 ml volumetric flask as described in the table below. (Calculate the new amounts if you use flasks of some other size.)
- 3.

Concentration of the standard	Amount of Mg- working solution (10mg/l)	Amount of 1M HCl	Amount of CsCl+LaCl ₃ solution
0.1 mg/l	1 ml	2 ml	0.5ml
0.3 mg/l	3 ml	2 ml	0.5ml
0.5 mg/l	5 ml	2 ml	0.5ml

Fill up to mark with milliQ water and mix well.

- Samples:

1. Filter the urine with filters, which are washed with milliQ water and use the 10 ml syringes.
2. Pipet the correct amount of sample, HCl and CsCl+LaCl solution into the volumetric flask, depending on the required dilution. Fill up to mark with milliQ water and mix well.

pH of the sample	Dilution	Amount of filtered urine	Amount of 1M HCl	Amount of CsCl+LaCl ₃	The size of volumetric flask
< 7	1:400	250 µl	2 ml	0.5 ml	100ml
> 8.5	1:20	2.5 ml	1 ml	0.25 ml	50 ml
7-8.5 (might be good)	1:200	0,5 ml	2ml	0.5 ml	100 ml

- Zero:

2(2)

Pipet 2 ml of 1M HCl + 0.5 ml of CsCl+LaCl solution into 100ml volumetric flask and fill up to mark with milliQ water. (Estimate how much you need, you will need it between each sample).

Calcium:

- Standards:
 1. Make the working solution (100mg/l) daily from calcium stock solution (1000mg/l) by pipetting 10ml of stock solution into 100 ml volumetric flask and fill up to mark with milliQ water.
 2. Prepare standards into 50 ml volumetric flask as advised in the table below (or calculate new amounts if you use flasks of some other size.)

Concentration of the standard	Amount of Ca – working solution (100 mg/l)	Amount of 1 M HCl	Amount of CsCl+LaCl ₃ solution
1 mg/l	0.5 ml	1 ml	2.5 ml
3 mg/l	1.5 ml	1 ml	2.5 ml
5 mg/l	2.5 ml	1 ml	2.5 ml

Fill up to mark with milliQ water and mix well.

- Samples:
 3. Filter the urine with washed filters as in Mg analysis.
 4. Pipet the correct amount of the sample and CsCl+LaCl solution into the volumetric flask, depending on the required dilution. Fill up to mark with milliQ water and mix well.

pH of the sample	dilution	amount of filtered urine	amount of 1M HCl	amount of CsCl+LaCl ₃ solution	The size of volumetric flask
< 7	1:50	1 ml	1 ml	2.5 ml	50 ml
> 8.5	1:20	2.5 ml	1 ml	2.5 ml	50 ml

- Zero

Pipet 2 ml of 1M HCl + 5ml of CsCl+ LaCl solution into the 100ml volumetric flask and fill up to mark with milliQ water. (Estimate how much you need, you will need it between each sample).

APPENDIX 2

Residuals of the test series

1(2)

Residuals Ca- new protocol 18.10.2011

First series			
Standard	Sig.	Conc.	Residuals
st1	0.51	0.042	-1.84E-06
st2	1.001	0.083	0.00025
st3	1.982	0.164	-0.00015
st4	2.96	0.245	-0.00030
st5	3.896	0.323	2.516E-05
st6	4.798	0.398	0.00018

Second series			
Standard	Sig.	Conc.	Residuals
st1	0.51	0.042	-3.208E-05
st2	1.007	0.083	1.796E-05
st3	1.991	0.165	-0.00013
st4	2.951	0.245	0.00038
st5	3.898	0.323	-0.00029
st6	4.804	0.398	5.028E-05

Third series			
Standard	Sig.	Conc.	Residuals
st1	0.512	0.042	-3.571E-05
st2	1.005	0.083	-0.00026
st3	1.994	0.165	0.000114
st4	2.963	0.246	0.00048
st5	3.886	0.322	-7.249E-05
st6	4.821	0.4	-0.00023

Residuals Mg – new protocol 20.10.2011

Serie1	sig.	conc.	Residuals
st1	0.04	0.057	-0.00052
st2	0.095	0.137	0.00037
st3	0.252	0.363	0.00056
st4	0.304	0.437	-0.00023
st5	0.404	0.581	-5.515E-05
st6	0.5	0.719	-0.00013

Serie2	sig.	conc.	Residuals
st1	0.04	0.057	-0.0003
st2	0.095	0.136	-0.00048
st3	0.251	0.363	0.0019
st4	0.304	0.437	-0.00035
st5	0.403	0.579	-0.00087
st6	0.501	0.721	5.13E-05

Serie3	sig.	conc.	Residuals
st1	0.04	0.057	-0.0007
st2	0.094	0.136	0.00067
st3	0.253	0.364	0.00011
st4	0.305	0.439	0.00037
st5	0.403	0.579	-0.00051
st6	0.5	0.719	5.77E-05